The experimental procedure pertaining to the present study “Antioxidative and antitumorigenic efficacy of protein fraction of *Cynodon dactylon* and its characterisation by *in silico* methods” are elaborated in this chapter.

The present study has been carried in five phases

**IN VITRO STUDIES**

**PHASE I**

**3.1** Assessment of protein content and characterisation of PFC

3.1.1 Evaluation of protein content of the dialysates of *Cynodon dactylon* in 10 – 100 % saturation of recrystallized ammonium sulphate

3.1.2 Characterisation of the selected PFC by native PAGE and SDS PAGE

**PHASE II**

**3.2** Assessment of antioxidative and antitumorigenic efficacy of PFC

3.2.1 Evaluation of free radical scavenging activity of PFC

3.2.1.1 DPPH scavenging activity

3.2.1.2 Nitric oxide scavenging activity

3.2.1.3 Hydrogen peroxide scavenging activity

3.2.2 Evaluation of antitumorigenic effect of PFC to DLA tumor cells

**IN VIVO STUDIES**

**PHASE III**

**3.3.** Assessment of antioxidative effect of PFC and silymarin in *CCl₄* challenged Swiss albino mice

3.3.1 Evaluation of the effect of PFC on the status of the liver

3.3.1.1 Effect of PFC on the Serum Glutamic Oxaloacetic Transaminase (SGOT) and on the Serum Glutamic Pyruvic Transaminase (SGPT) activities
3.3.2 Evaluation of the effect of PFC on the activities of hepatic enzymic antioxidants
   3.3.2.1 Effect of PFC on superoxide dismutase (SOD) activity
   3.3.2.2 Effect of PFC on catalase (CAT) activity
   3.3.2.3 Effect of PFC on glutathione peroxidase (GPx) activity

3.3.3 Evaluation of the effect of PFC on the levels of hepatic non enzymic antioxidants
   3.3.3.1 Effect of PFC on the levels of vitamin A
   3.3.3.2 Effect of PFC on the levels of vitamin C
   3.3.3.3 Effect of PFC on the levels of vitamin E
   3.3.3.4 Effect of PFC on the levels of reduced glutathione (GSH)

3.3.4 Evaluation of the effect of PFC on the levels of TBARS

PHASE IV

3.4 Assessment of antitumorigenic effect of PFC in DLA induced Swiss albino mice
   3.4.1 Evaluation of antitumorigenic effect of PFC

3.4.2 Evaluation of effect of PFC on the activities of hepatic enzymic antioxidants
   3.4.2.1 Effect of PFC on superoxide dismutase (SOD) activity
   3.4.2.2 Effect of PFC on catalase (CAT) activity
   3.4.2.3 Effect of PFC on glutathione peroxidase (GPx) activity

3.4.3 Evaluation of the effect of PFC on the levels of hepatic non enzymic antioxidants
   3.4.3.1 Effect of PFC on the levels of vitamin A
   3.4.3.2 Effect of PFC on the levels of vitamin C
   3.4.3.3 Effect of PFC on the levels of vitamin E
   3.4.3.4 Effect of PFC on the levels of reduced glutathione (GSH)

3.4.4 Evaluation of the effect of PFC on the levels of TBARS

3.4.5 Evaluation of the effect of PFC on the histological pattern of liver in DLA induced mice
**IN SILICO STUDIES**

PHASE V

3.5 Prediction of structures and models of PFC

3.5.1 Primary structure of PFC using PROTPARAM tools in ExPASY

3.5.2 Secondary structure of PFC using GOR tool in ExPASY

3.5.3 Molecular models of PFC using the template 1 KRR

3.6 STATISTICAL ANALYSIS

**IN VITRO STUDIES**

PHASE I

3.1 Assessment of protein content and characterisation of PFC

Selection of the plant material

The fresh plant material *Cynodon dactylon* (Arugampul) was collected from the pesticide free area, washed thoroughly to remove the dust particles, blotted dry between filter papers. The leaves were homogenized with phosphate buffered saline (PBS) at 4ºC to obtain 20 % homogenate. The homogenate was strained through 8 layers of cotton gauze and centrifuged at 5000 rpm for 10 minutes at 4º C. The supernatant so obtained was used for the ammonium sulphate fractionation of proteins (Plate 1).

**PLATE 1**

*Cynodon dactylon* (L.) Pers.

<table>
<thead>
<tr>
<th>Common name</th>
<th>Arugampul</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class</td>
<td>Monocotyledons</td>
</tr>
<tr>
<td>Subclass</td>
<td>Glumaceae</td>
</tr>
<tr>
<td>Order</td>
<td>Poales</td>
</tr>
<tr>
<td>Family</td>
<td>Poaceae</td>
</tr>
<tr>
<td>Genus</td>
<td><em>Cynodon</em></td>
</tr>
<tr>
<td>Species</td>
<td><em>dactylon</em></td>
</tr>
</tbody>
</table>
3.1.1 Evaluation of protein content of the dialysates of *Cynodon dactylon* in 10 - 100% saturation of recrystallized ammonium sulphate

10 - 100% ammonium sulphate fractionations of proteins was carried out using recrystallized ammonium sulphate and the precipitate obtained by this method was dissolved in 0.01 M PBS by the method of Jayaraman (1981) (Appendix I). The protein content of all the fractions of the precipitate obtained after dialysis (Appendix II) was estimated using the method of Shakir *et al.*, 1994 (Appendix III).

3.1.2 Characterisation of the selected PFC by native PAGE and SDS PAGE

The selected 70% PFC was characterized by NATIVE PAGE (Appendix IV) and SDS PAGE (Appendix V).

**PHASE II**

3.2 Assessment of antioxidative and antitumorigenic efficacy of PFC

3.2.1 Evaluation of free radical scavenging activity of PFC

3.2.1.1 DPPH scavenging activity

The antioxidant activity in PFC were performed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging method (Mensor *et al.*, 2001) as expressed in Appendix VI.

3.2.1.2 Nitric oxide scavenging activity

The antioxidant activities in PFC were performed by determination of scavenging of nitric oxide (Green *et al.*, 1982) as expressed in Appendix VII.

3.2.1.3 Hydrogen peroxide scavenging activity

The antioxidant activities in PFC were performed by hydrogen peroxide scavenging assay (Ruch *et al.*, 1989) as expressed in Appendix VIII.
3.2.2 Evaluation of antitumorigenic effect of PFC to DLA tumor cells

*In vitro* cytotoxic studies were carried out to find out the *in vitro* antitumorigenic effect of the protein fraction in 70% saturation of ammonium sulphate precipitation of *Cynodon dactylon* against DLA cells and also to determined 50 per cent effective concentration (EC$_{50}$) by trypan blue exclusion method of Salomi and Panikkar (1989) (Appendix IX). The fraction which showed EC$_{50}$ at minimum concentration was selected for the *in vivo* studies.

**IN VIVO STUDIES**

**PHASE III**

3.3 Assessment of antioxidative effect of PFC and silymarin in CCl$_4$ challenged swiss albino mice

**Experimental animals**

Swiss albino mice of 5 to 7 weeks (20 to 25 g) were obtained from animal breeding station, Kerala Agricultural University, Thrissur. The mice were acclimatized to laboratory conditions for 15 days before the commencement of experiments. All procedures described were reviewed and approved by the University Animals Ethical Committee (Reg. No.623/02/b/CPSCSEA).

The mice were maintained under standard laboratory conditions. They were housed in cages bottomed with husk, maintained at room temperature and provided with the standard feed pellets which are commercially available and with water ad libitum. After randomization into groups, the mice were acclimatized for a period of two weeks in the environment.

To assess the antioxidative effect of PFC, the Swiss albino mice were divided into seven groups with six mice in each and were treated intraperitoneally for 21 days.
Group 1 received 100μl of PBS everyday and served as the vehicle control for Group 3.

Group 2 received 100μl of paraffin oil (PO) everyday and served as the vehicle control for the groups 4 and 5.

Group 3 received 40 µg of PFC (EC$_{50}$) in 100μl of PBS.

Group 4 received standard antioxidant silymarin (SMN) 25mg / kg body weight suspended in 100μl of paraffin oil (Letteron et al., 1990).

Group 5 received 1 ml / kg body weight of CCl$_4$ suspended in 100μl of paraffin oil (Gerhardvogel, 2002).

Group 6 received 25mg standard antioxidant silymarin / kg body weight in 100μl of paraffin oil + 1 ml / kg body weight of CCl$_4$ suspended in 100μl of paraffin oil.

Group 7 received 40 µg of PFC (EC$_{50}$) in 100μl of PBS + 1 ml / kg body weight of CCl$_4$ suspended in 100μl of paraffin oil.

Animals grouped under 5, 6 and 7 received one acute dose of CCl$_4$ before 24 hrs of the experimental tenure (21 days) along with the scheduled regimen. At the end of the experimental tenure, the animals were kept overnight fasting, sacrificed and the livers were excised. Blood was collected, serum was separated and used for the assay of the liver marker enzymes.

### 3.3.1 Evaluation of the effect of PFC on the status of the liver

To assess the status of the liver, the activities of the liver marker enzymes – SGOT and SGPT were carried out.

Serum Glutamic Oxaloacetic Transaminase (EC. 2.6.1.1) was assayed by the method of Reitman and Frankel, 1957 as given in Appendix X.
Serum Glutamic Pyruvic Transaminase (EC. 2.6.1.2) was assayed by the method of Reitman and Frankel, 1957 as in Appendix XI.

3.3.2 Evaluation of the effect of PFC on the activities of hepatic enzymic antioxidants

The following parameters were analyzed in the liver homogenate without elapse of time to avoid variations.

3.3.2.1 Superoxide dismutase (SOD, EC.1.15.1.1)

The activity of superoxide dismutase was estimated by the method of Misra and Fridovich (1972) as given in Appendix XII.

3.3.2.2 Catalase (CAT, EC.1.11.1.6)

The catalase activity was assessed by the method of Luck, 1974 as in Appendix XIII.

3.3.2.3 Glutathione Peroxidase (GPx, EC1.11.1.7)

The activity of glutathione peroxidase was determined by the method of Rotruck et al., (1973) as in Appendix XIV.

3.3.3 Evaluation of the effect of PFC on the levels of hepatic non enzymic antioxidants

3.3.3.1 Vitamin A (Retinol)

Vitamin A was estimated by the method of Bayfield and Cole (1980) as shown in Appendix XV.

3.3.3.2 Vitamin C (Ascorbic acid)

Vitamin C was assessed by the method of Roe and Kuether (1953) as in Appendix XVI.
3.3.3.3 Vitamin E (α-tocopherol)

The α-tocopherol content was determined by the method of Rosenberg (1992) as given in Appendix XVII.

3.3.3.4 Reduced Glutathione (GSH)

Reduced glutathione, the substrate of Glutathione-S-Transferase was assessed by the method of Moron et al., (1979) as in Appendix XVIII.

3.3.4 Evaluation of the effect of PFC on the levels of TBARS

Levels of TBARS was assessed by the method of Nichans and Sameulson (1963) as given in Appendix XIX.

PHASE IV

3.4 Assessment of antitumorigenic effect of PFC in DLA induced swiss albino mice

Propagation of DLA tumor cells

Dalton’s Lymphoma Ascite (DLA) tumor cells were procured from Amala Cancer Research Centre, Thrissur, Kerala. The mice were acclimatized for two weeks and cells were propagated by intraperitoneal transplantation of $1 \times 10^6$ cells in $100 \, \mu l$ of PBS. After 10-15 days, the cells were drawn from the intraperitoneal cavity and used for the *in vitro* cytotoxic and *invivo* antitumorigenic studies.

*In vivo* studies were carried out by the intraperitoneal administration of $EC_{50}$ of 70 per cent ammonium sulphate protein fraction of *Cynodon dactylon* (PFC) to examine the antitumorigenic activity. The antitumor activity were also determined by administrating $EC_{50}$ of PFC at different stages of tumor induction (initiation, post- initiation and entire period of tumorigenesis) in DLA cells induced Swiss albino mice (Plate 2).
3.4.1 Evaluation of antitumorigenic effect of PFC

The anti tumorigenic potential of the PFC was assessed by evaluating the ILS by the method of Geran et al., 1972 (Appendix XX).

Based on the findings of ILS, the treatment period of the experiment was designed as 15 days treatment period and 90 days treatment period. The animals were administered intraperitoneally with one acute dose of $1 \times 10^6$ DLA tumor cells in 0.1 ml of PBS on various days along with PFC so as to assess the tumorigenic potential of the PFC. Animals were grouped into six with 12 mice in each.

- Group 1 received 0.1ml of PBS everyday and served as the vehicle control
- Group 2 received EC$_{50}$ of PFC (40 µg in 100µl of PBS)
Group 3 received one acute dose of $1 \times 10^6$ DLA tumor cells in 0.1ml of PBS on the first day of the experimental tenure and served as the DLA control.

Group 4 received 40 µg of PFC ($EC_{50}$) one week before the DLA tumor cell administration (Initiation period).

Group 5 received 40 µg of PFC ($EC_{50}$) one week after the DLA tumor cell administration (Post initiation period).

Group 6 received 40 µg of PFC ($EC_{50}$) and the DLA tumor cell administration on the first day and PFC administration alone continued for 90 days (Entire period).

At the end of the 15th and 90th days, the animals were kept overnight fasting and sacrificed. Livers were excised and used for the assay of the activities of enzymic antioxidants (SOD, CAT and GPx) the levels of non enzymic antioxidants (vitamin A, C and E as well as GSH) and levels of TBARS at different stages of tumor induction (initiation, post initiation and entire period of tumorigenesis). All these estimations of enzymic, non enzymic and TBARS were carried as described in Phase III.

3.4.2 Evaluation of the effect of PFC on the histological pattern of liver in DLA induced mice

Histological architecture of the hepatocytes were carried out by the method of Culling, (1979) as per Appendix XXI.

IN SILICO STUDIES

PHASE V

3.5 Prediction of structures and models of PFC

The amino acid sequence of the isolated protein was done by Edman degradation method (Appendix XXII). Having found the sequence of aminoacids by protein sequencing methods in the wet lab, sequence was submitted to the
ExPASY proteomics tools for the primary and secondary structure prediction [http://www.expasy.org/tools#primary].

3.5.1 Primary structure of PFC using PROTPARAM tools in ExPASY
3.5.2 Secondary structure of PFC using GOR tool in ExPASY
3.5.3 Molecular models of PFC using the template 1 KRR

3.6 STATISTICAL ANALYSIS

The data were statistically analyzed by one-way ANOVA for *in vivo* antioxidative studies. The results were analyzed in antitumorigenesis protocol using one-way analysis of variance (ANOVA) including DLA for 15 days and excluding DLA for 90 days and two-way analysis of variance (ANOVA) for both 15 and 90 days excluding DNA for the tumorigenesis protocol. The findings were considered significant if P<0.05.